Evaluation of Lightmix Mycoplasma macrolide assay for detection of macrolide-resistant Mycoplasma pneumoniae in pneumonia patients

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Abstract: OBJECTIVES Rapid detection of macrolide resistance-associated mutations in Mycoplasma pneumoniae is crucial for effective antimicrobial treatment. We evaluated the Lightmix Mycoplasma macrolide assay for the detection of point mutations at nucleotide positions 2063 and 2064 in the 23S ribosomal RNA (rRNA) gene of M. pneumoniae that confer macrolide resistance. METHODS Samples from 3438 patients with a respiratory tract infection were analysed by M. pneumoniae real-time PCR, and 208 (6%) of them were tested positive. In this retrospective study, 163 M. pneumoniae real-time PCR-positive samples were analysed by the Lightmix assay, and results were compared to targeted 23S rRNA sequencing. RESULTS Macrolide-resistant M. pneumoniae were found in 15 (9%) of 163 retrospectively analysed samples. The Lightmix assay showed a sensitivity of 100% (95% confidence interval, 78.2-100) and a specificity of 100% (95% confidence interval, 97.5-100) as the detected M. pneumoniae genotype (148 wild type and 15 non-wild type) was confirmed by 23S rRNA sequencing in all samples. CONCLUSIONS The Lightmix assay is an easy-to-use and accurate molecular test that allows rapid determination of macrolide resistance in M. pneumoniae.

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Research note

Evaluation of Lightmix *Mycoplasma* macrolide assay for detection of macrolide-resistant *Mycoplasma pneumoniae* in pneumonia patients

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ABSTRACT

Objectives: Rapid detection of macrolide resistance–associated mutations in *Mycoplasma pneumoniae* is crucial for effective antimicrobial treatment. We evaluated the Lightmix *Mycoplasma* macrolide assay for the detection of point mutations at nucleotide positions 2063 and 2064 in the 23S ribosomal RNA (rRNA) gene of *M. pneumoniae* that confer macrolide resistance.

Methods: Samples from 3438 patients with a respiratory tract infection were analysed by *M. pneumoniae* real-time PCR, and 208 (6%) of them were tested positive. In this retrospective study, 163 *M. pneumoniae* real-time PCR–positive samples were analysed by the Lightmix assay, and results were compared to targeted 23S rRNA sequencing.

Results: Macrolide-resistant *M. pneumoniae* were found in 15 (9%) of 163 retrospectively analysed samples. The Lightmix assay showed a sensitivity of 100% (95% confidence interval, 78.2–100) and a specificity of 100% (95% confidence interval, 97.5–100) as the detected *M. pneumoniae* genotype (148 wild type and 15 non–wild type) was confirmed by 23S rRNA sequencing in all samples.

Conclusions: The Lightmix assay is an easy-to-use and accurate molecular test that allows rapid determination of macrolide resistance in *M. pneumoniae*. **K. Wagner, Clin Microbiol Infect 2019;25:383.e5–383.e7**

Introduction

Mycoplasma pneumoniae is a common pathogen causing respiratory tract infections, particularly in children and young adults [1]. Macrolides are used as empiric first-line treatment of *M. pneumoniae* infections. However, their extensive use led to the rapid worldwide emergence of clinically macrolide-resistant *M. pneumoniae* strains [2,3]. The main mechanism of macrolide resistance in *M. pneumoniae* is nucleotide substitutions at positions A2063 and A2064 in domain V of the 23S ribosomal RNA (rRNA) gene (*M. pneumoniae* numbering; corresponds to nucleotide positions 2058 and 2059 in *Escherichia coli*) [4].

The aim of this study was to evaluate the reliability and accuracy of the commercially available molecular Lightmix assay to detect macrolide-resistant *M. pneumoniae* in respiratory specimens of patients with pneumonia.

Methods

Clinical specimens and M. pneumoniae real-time PCR

All respiratory specimens ($N = 3438$) used in this study were sent to the Institute of Medical Microbiology, University of Zurich, between January 2014 and December 2017 for the detection of respiratory bacterial pathogens, including *M. pneumoniae,* from secondary or tertiary care facilities, and were recovered from patients with respiratory tract infection symptoms. Isolation of chromosomal DNA and *M. pneumoniae* real-time PCR (RT-PCR) detection (henceforward termed *M. pneumoniae* RT-PCR) was performed on all 3438 respiratory specimens as described previously [5–7].

Lightmix Mycoplasma macrolide assay

Chromosomal DNA of respiratory specimens was stored at −20°C. Before conducting this retrospective study, the DNA concentration of all *M. pneumoniae* RT-PCR–positive samples was determined using the Qubit dsDNA HS kit (Thermo Fischer

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Scientific, Schlieren, Switzerland). Samples with a DNA concentration of >0.5 ng/μL were analysed by the Lightmix *Mycoplasma* macrolide assay (TIBMolbiol, Berlin, Germany) (Supplementary Fig. S1). The Lightmix assay consists of a RT-PCR amplifying a 117 bp fragment of the 23S rRNA gene and subsequent melting curve analysis. The assay was performed on a LightCycler 480-II instrument (Roche, Rotkreuz, Switzerland). A melting temperature (T_m) greater 66°C in the Lightmix assay indicated a wild-type genotype, while a T_m lower than 62°C indicated the presence of a point mutation at position A2063 or 2064 in the amplified 23S rRNA gene, rendering the detected *M. pneumoniae* macrolide resistant. To verify the genotype detected by the Lightmix assay, all 163 23S rRNA amplicons were sequenced and analysed with respect to resistance-conferring point mutations.

23S rRNA RT-PCR amplicon sequencing

The 23S rRNA RT-PCR products were cleaned using AmpPure XP beads (Beckman Coulter, Nyon, Switzerland), and library preparation was done with the QIAseq FX DNA library preparation kit (Qiagen, Hilden, Germany) following the producers' recommendations except omitting the fragmentation step. Sequencing library quality and size distribution was analysed on a fragment analyser (Advanced Analytical Technologies, Heidelberg, Germany) using the Fragment Analyzer 474 HS NGS kit. Sequencing libraries were pooled in equimolar concentrations and paired-end sequenced (2 × 150 bp) on an Illumina MiSeq platform (Illumina, San Diego, CA, USA). Reads of the 23S rRNA gene sequences of the *M. pneumoniae* isolates were mapped to the 23S rRNA gene of *M. pneumoniae* strain M129 using CLC Genomic Workbench software (Qiagen).

Data analysis

A chi-square test of independence was performed to examine the associations among age, proportion of *M. pneumoniae* infections and macrolide resistance. All statistical data analysis and data visualization were performed using R software [8].

Ethics statement

This retrospective study was conducted in accordance with the Declaration of Helsinki and national and institutional standards. The act on medical research involving human subjects does not apply to this study. The study was approved by the ethics committee of the canton of Zurich, Switzerland (Req-2018-00473).

Results

Overall, 208 of the 3438 respiratory specimens were tested positive by *M. pneumoniae* RT-PCR. In 45 of 208 *M. pneumoniae*–positive samples, chromosomal DNA was degraded as a result of prolonged freezing (<0.5 ng/μL DNA; $n = 7$), or samples were no longer available for retrospective testing ($n = 38$) via the Lightmix assay. Of the remaining 163 samples, 148 (91%) showed a wild-type genotype in the Lightmix assay, while 15 (9%) displayed a mutation at nucleotide position A2063 or A2064 in the 23S rRNA gene conferring resistance to macrolides (Table 1, Supplementary Fig. S1).

Isolates classified as *M. pneumoniae* wild type in the Lightmix assay did not show a mutation in the domain V of the 23S rRNA gene, as verified by 23S rRNA gene amplicon sequencing. For all samples that showed a shifted melting temperature ($T_m < 62°C$; $n = 15$) in the Lightmix assay, sequencing confirmed a mutation either at nucleotide position 2063 or 2064. We found nine A2063G mutations, five A2064G mutations and one A2063C mutation (Table 1). This resulted in a sensitivity of 100% (95% confidence interval, 78.2–100) and a specificity of 100% (95% confidence interval, 97.5–100) of the Lightmix assay.

Interestingly, in our study, we found that the median age of patients with a *M. pneumoniae* infection was 15 years, while the median age of patients with macrolide-resistant *M. pneumoniae* infections was 47 years (Supplementary Fig. S2). There was a clear association between the age of patients and *M. pneumoniae* infection (χ^2 (5, $n = 3217$) = 328.0, $p < 0.001$) with the highest proportion of *M. pneumoniae* infections in patients aged 0 to 20 years (Supplementary Fig. S3(A)). Interestingly, among patients with *M. pneumoniae* infections, younger patients showed infections with macrolide-susceptible *M. pneumoniae,* but macrolide-resistant *M. pneumoniae* infections were more prevalent in patients aged >31 years (χ^2 5, $n = 211$) = 12.1, $p < 0.05$), with the strongest association in patients aged >50 years (Supplementary Fig. S3(B)).

Discussion

In this retrospective study, the Lightmix *Mycoplasma* macrolide assay was evaluated on respiratory specimens of pneumonia patients and showed high specificity and sensitivity for the detection of point mutations in the 23S rRNA gene conferring macrolide resistance in *M. pneumoniae.*

In this study, a macrolide resistance rate of 9% was found in *M. pneumoniae* when analysing samples from patients treated at hospitals in Zurich, Switzerland. Prevalence of macrolide resistance in *M. pneumoniae* differs in Europe and has been previously reported as being 0 to 3% in Germany, the Netherlands and Switzerland [9–11], 9.8% in France [12] and 26% in Italy [13]. Differences in observed resistance rates in this study compared to previous studies conducted in Switzerland [9] may be due to two main factors. Firstly, the respiratory specimens analysed in this study are mainly obtained from patients treated at secondary or tertiary care facilities. Therefore, the patient population may be selected in terms of progression of disease, severity of pneumonia and proportion of persistent or recurrent *M. pneumoniae* infections; further, patients are often already receiving antibiotic therapy when samples are retrieved for diagnostic procedures. Secondly, the age distribution of the patient population is very broad (1 to 98 years old), and therefore resistance rates may differ from studies that primarily focused on *M. pneumoniae* infection and macrolide resistance in children and young adults [9]. This notion is supported by the fact that we found a

Table 1
Retrospective analysis of respiratory specimens by Lightmix assay and confirmation of detected *Mycoplasma pneumoniae* genotype by 23S rRNA amplicon sequencing

M. pneumoniae by RT-PCR		Genotype not determined	Retrospective analysis by Lightmix assay		Genotype determined by 23S rRNA sequencing	
Negative	Positive		WT	Non-WT	WT	Non-WT
3230 (94%)	208 (6%)	45	148 (91%)	15 (9%)	148	A2063C (1 ×), A2063G (9 ×), A2064G (5 ×)

rRNA, ribosomal RNA; RT-PCR, real-time PCR; WT, wild type.

higher proportion of macrolide-resistant *M. pneumoniae* infections in patients aged >31 years. This indicates that macrolide resistance may be associated with age and possibly long-term antibiotic consumption, as macrolides are among the most commonly prescribed antibiotics in Switzerland [14].

This study has some limitations. It was designed as a single-centre laboratory-based, retrospective evaluation study with extracted DNA from clinical respiratory specimens. We found a high macrolide resistance rate in *M. pneumoniae* in the investigated patient population. This would need to be further addressed in future studies, including patients from primary care facilities. Thus, continuous surveillance is crucial to monitor macrolide resistance in *M. pneumoniae* and to further investigate its epidemiology in Switzerland.

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Transparency Declaration

Lightmix kits for the analysis of 100 samples were donated by TIBMolbiol, Berlin, Germany. For the analysis of the remaining samples, Lightmix kits were purchased from TIBMolbiol by the Institute of Medical Microbiology, University of Zurich. All authors report no conflicts of interest relevant to this article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cmi.2018.10.006.

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